MINOR RESEARCH

What can we learn from comparing Giants with Miniatures – The role of *NOTCH4* signalling in the canine growth plate and chondrogenic differentiation of canine bone marrow derived mesenchymal stromal cells.

Michelle Teunissen
Student number: 3516342
Supervisors: Alberto Miranda-Bedate, Marianna Tryfonidou, Frank Riemers

Abstract
At this moment there are many challenges in the regeneration of large bone defects. An interesting alternative to the current treatment standards could be the use of mesenchymal stem cells (MSCs). These cells produce bone by endochondral bone formation, thereby overcoming the limitations that arise with the use of bone grafts. In this project, we used next generation sequencing to study differences in the gene expression profile of the growth plate of Great Danes (GD), a large breed dog, and Miniature Poodles (MP), a small breed dog, in order to find new targets that could enhance the pace of endochondral bone formation.
We found that *NOTCH4* was highly upregulated (FC: 490) in the proliferative zone of the GD growth plate compared to the MP, although absolute transcript counts were low (7 counts (GD) vs 0 counts (MP)). Using immunohistochemistry to validate the difference in NOTCH4 expression at the protein level, we found significantly more NOTCH4 protein in the proliferative and reserve zone of GD growth plate compared to the MP growth plate. Interestingly, NOTCH4 expression was mainly found in the chondrocytes surrounding the blood vessels. The next step of the project was to induce expression of *NOTCH4* in bone marrow derived MSCs in order to investigate the role of *NOTCH4* during endochondral bone formation. Unfortunately, none of the used growth factors or small molecules were able to induce *NOTCH4* expression. These findings suggests that although *NOTCH4* might play an interesting role in the canine growth plate, further research is necessary.

Introduction
Outline of the problem
In the field of bone regenerative medicine there are still many challenges in the regeneration of large bone defects and the repair of complicated fractures. Although there's a wide range in bone regenerative therapies, including bone grafts, these current therapies have limitations and can even be affected by complications.
The state of the art treatment at this moment is the use of autografts (Dimitriou, Jones, McGonagle, & Giannoudis, 2011). Autografts are histocompatible and non-immunogenic, and offer the essential osteoinductive, osteogenic and osteoconductive properties. However, the use of autografts requires a second surgery to harvest the material which entails all the surgery risks. In addition, the amount of available donor tissue is limited in small patients. An alternative for autografts are allografts or xenografts (Dimitriou et al., 2011). The use of these therapies may solve the issues of limited graft tissue, but is hampered by graft rejection.
Another major problem in large bone reconstructions is the generation of adequate blood supply, which is needed for the high oxygen demand of the osteoblasts. Due to the lag-time of vascularization of the large segments, hypoxia and nutrient deficiency occur and result into decreased proliferation of the osteoblasts and into reduction of the quality and quantity of the matrix produced by the osteoblasts (Utting et al., 2006).

Bone defects heal secondary by endochondral bone formation, a process in which precursor cells differentiate towards the chondrogenic lineage and produce a cartilage matrix. This matrix is later replaced by bone after which the bone defect is repaired without the formation of scar tissue.
An alternative for bone grafts may therefore be the use of mesenchymal stem cells (MSCs). MSCs can produce bone through the endochondral process by differentiating to chondrocytes. Because chondrocytes can withstand low oxygen tension, the use of MSC's provides an opportunity to overcome the obstacles of inadequate vascularization and the limited availability of graft tissue (Gawlitta et al., 2010). Current bone regenerative strategies concentrate on the combination of MSC's, supportive scaffolds and growth factors to accelerate direct bone formation but could also be used to stimulate indirect bone formation, which is often seen in the clinical experiments. Members of the bone morphogenetic proteins (BMPs), rhBMP-2 and rhBMP-7 , are already being used to enhance fracture healing in a clinical setting (Lissenberg-Thunnissen, de Gorter, Sier, & Schipper, 2011). Although various animal and in vitro studies have demonstrated the positive effects of BMPs, the results of clinical trials have recently been subject to discussion (Carragee, Hurwitz, & Weiner, 2011; Garrison et al., 2007). For example, the use of rBMP2 has been related to complications and BMP-7 has been shown not to have an additive effect over autograft in achieving fusion of vertebrae (Agarwal, Williams, Umscheid, & Welch, 2009). Therefore the search for new bone regenerative strategies is still going.

Variation in height

New bone regenerative strategies could be found by studying the physiologic process of endochondral bone formation. In longitudinal growth, endochondral bone formation occurs in a spatial and organized manner and hence is a valuable model for studying the different pathways that contribute to natural growth and to the pace of endochondral bone formation. Historically, the regulation of endochondral bone formation was studied by focusing on mutations that cause dysregulation of growth, which are rare in the general population and may cause other problems unrelated to it. In this respect, studying the more natural phenomenon of variation in adult height, and hence variation in growth, gives insight into the role of the different endocrine and local pathways that influence bone formation.

Height is a highly heritable but complex polygenic trait and therefore large population studies are necessary in order to discover the causative genes (Lettre, 2011). In genome wide association studies (GWAS) evaluating the role of genes in natural height variation, candidate genes are selected based on their known function or presence in pathways related to postnatal growth. Recent meta-analysis of over 50 GWAS concerning over 183.000 individuals (GIANT GWAS) identified more than 180 loci to be associated with adult height (Lango Allen et al., 2010). Nevertheless, all these data combined only explained 10% of the heritability of height in humans. In addition, using information of single nucleotide polymorphisms (SNPs) in GWAS studies frequently results in larger genomic regions correlated with the phenotype. This correlation can easily be caused by variations in neighboring genes as well as transcription regulating regions.

In order to overcome this, Lui et al., (2012) pursued an integrative approach and performed two micro-arrays in which the genetic profiles from the murine growth plate were compared with three different soft tissues (lung, kidney and heart) during growth and spatially or temporally regulated genes in the different zones of the rat growth plate were identified (Lui et al., 2012). From the 420 genes that met their criteria, 38 corresponded with the 180 loci of the GIANT GWAS , and 22 of these represented the gene closest to the SNP identified by the GWAS (Lui et al., 2012). This overlap shows a strong enrichment of the growth plate genes in the GIANT GWAS list. Such integrative approaches help unravel the signaling pathways related to height variation. However, one of the

limitations of this elegantly performed study is that mice and rats do not share growth plate physiology with humans as they do not close their growth plates at skeletal maturity (Kember & Sissons, 1976).

Canine variation in height

In this respect, the dog has been an invaluable model in identifying genes determining size Canine genetic studies identified SNPs related to the Insulin-like Growth Factor I (*IGF1*) gene and the IGF1 receptor (*IGF1R*) gene which were associated with small size in a diverse set of small dog breeds(Hoopes, Rimbault, Liebers, Ostrander, & Sutter, 2012; Sutter et al., 2007). More SNPs were found in other genes (such as *HMAG2* and *SMAD2*) and it was shown that a combination of the variations found in the *HMGA2, SMAD2, IGF1,IGF1R*, growth hormone receptor (*GHR*) and Stanniocalcin-2 (*STC2*) genes explain around 50% of the variance in body size of dogs (Rimbault et al., 2013). However, these SNPs only explain small size and not the enhanced growth of large dog breeds.

Background of the project

The main focus of this project is to study the local mechanisms that regulate the pace of endochondral bone formation in order to find new bone regenerative strategies. In the past, a two color microarray analysis was performed to compare the growth plate of large breed dogs (Great Danes) with the growth plate of small breed dogs (Miniature Poodles). Microarray analysis showed 2981 unique differential expressed genes of which 1202 were upregulated and 1779 were down regulated in the GD compared to MP, among these genes were well known longitudinal growth contributors such as *SOX9, IHH, BMP-2* and *-6*, collagen type XIα, and aggrecan.

However, the microarray was performed using a mixture of the whole growth plate and adjacent primary spongiosa bone. A better understanding of the pathways regulating the pace of endochondral bone formation can only achieved by investigating the separate zones of the growth plate. Therefore next generation sequencing (NGS) of the separate growth plate zones of GD and MP was performed to identify DE-genes in a quantitative manner and define the differential spatial expression pattern. In addition to the discovery of DE genes, the NGS allows the discovery of new genes and transcripts and measure transcript expression at the same time. In contrast to microarray analysis, the NGS allows as well an unbiased examination of the transcriptome and the detection of novel and alternative spliced transcripts while measuring the expression of these transcripts and will do so with an enhanced sensitivity and at least an equal accuracy.

Notch4

One of the interesting findings of the NGS performed by this group was the discovery of the differential expression of *NOTCH4*. *NOTCH4* is highly upregulated (Fold Change of 490) in the proliferative zone of the growth plate of Great Danes compared with miniature poodles and is therefore an interesting candidate for the regulation of the pace of endochondral bone formation.

Notch4 is a cell surface receptor that consists of an extracellular domain (containing multiple (EGF)-like repeats), a transmembrane domain and an intracellular domain (Ntziachristos, Lim, Sage, & Aifantis, 2014). The Notch pathway, consisting of 4 Notch receptors including Notch4, is activated upon interactions with ligands such as Delta-like and Jagged, which are also transmembrane proteins containing EGF-like repeats. In mammals, there are three Delta-like ligands (Dll1, Dll3, and Dll4) and two Jagged ligands (Jag1 and Jag2). Upon ligand interaction, the intracellular portion of the Notch receptor (termed ICN) is cleaved and translocates into the nucleus to mediate target gene activation. Despite the overall similarities between the receptors, the differences in the ligand-binding

extracellular domains and the transactivation intracellular domains lead to distinct ligand affinities and capacities to activate downstream transcription.

Not much is known about the function of *NOTCH4*. The gene is associated at this moment with schizophrenia (Shayevitz, Cohen, Faraone, & Glatt, 2012) and breast cancer (Nagamatsu et al., 2014) and it is thought to influence the regulation of angiogenesis (Kume, 2012). In addition, studies show that Notch4 inhibits Notch1 signaling (James et al., 2014).

Notch signaling in skeletal development

Notch signaling has emerged as an important regulator of skeletogenesis with multiple roles in somitogenesis, chondrogenesis and osteoclastogenesis. Murine *in vitro* studies found that *Notch1* is expressed in early mesenchymal condensations were it promotes chondrogenic specification. However, over-expression of the Notch1 intra cellular domain or Dll1 inhibits chondrogenic proliferation and differentiation (Mead & Yutzey, 2012)(Mead & Yutzey, 2012). Moreover, although Notch signaling is necessary for the function as a trigger required to prime human MSCs for chondrogenesis (Oldershaw & Hardingham, 2010) , Notch1 has shown to have a strong inhibitory effect on both differentiation and proliferation of human MSCs when activated (Watanabe et al., 2003).

Notch4 expression was found in the rounding proliferative cells, the final 2–3 layers of flattened prehypertrophic chondrocytes and in the majority of hypertrophic chondrocytes in the growth plates of mice (Hayes, Dowthwaite, Webster, & Archer, 2003). However, regarding the role of Notch4 in the growth plate, not much is known.

The purpose of this project was to validate the found upregulation of *NOTCH4* in the proliferative zone of the GD compared with the MP as found with NGS. In order to investigate the distribution of the NOTCH4 protein, an immunohistochemical staining for NOTCH4 was performed on whole growth plate samples of GD and MP. In addition, in order to evaluate the possibility of using Notch4 treated BM-MSC for new bone regenerative therapies, it was attempted to stimulate the expression of *NOTCH4* in BM-MSC and AC with the growth factors Fibroblast Growth Factor 2 (FGF-2), and/or Dexamethasone or the small molecules; Withaferin A, TPA, and Luteolin, which were chosen based on the literature.

In the study of *Wu et. al.* (2007) it was demonstrated that *Notch4* transcription could be activated in endothelial cells of mouse yolk sac origin with cortisol (1 µg/ml), epidermal growth factor (EGF, 10 ng/ml) and or FGF-2 (10 ng/ml) with heparin (10 µg/ml) as a cofactor (Wu & Bresnick, 2007). This could be repeated in multipotent mouse embryonic 10T1/2 and also with dexamethasone (30nM and 300 nM) synergized with FGF-2 (Wu & Bresnick, 2007).
Withaferin A (WA) is small-molecule constituent of the ayurvedic medicine plant Withania somnifera. It has shown efficacy against cultured human breast cancer cells (Thaiparambil et al., 2011). The study of Lee et. al. (2012) investigated the effect of different concentrations of WA (2 and 4 µM) on the Notch signaling pathway in human breast cancer cells and found that while WA treatment resulted in a decrease in levels of transmembrane as well as cleaved (active) Notch1, the levels of cleaved Notch4 were increased markedly (Lee, Sehrawat, & Singh, 2012). TPA is a tumor-promoting drug that activates transcription of a number of genes that include a TPA response element including AP-1, which in its turns regulates the promotor activity of NOTCH4 among others (Canettieri et al., 2009; Eferl & Wagner, 2003; Wu et al., 2005). In contrast to the two other small molecules, Luteolin is a ribosomal S6 kinase (RSK) inhibitor that suppresses Notch4 expression in a

concentration of 50 µM after 48 hours by inhibiting Y-box binding protein-1 (YB-1), a protein that binds to the Notch4 promoter and increases its expression (Reipas et al., 2013).

Materials and Methods

Specimens

The growth plate tissue samples were obtained from animals used in *in vivo* experiments described elsewhere (Tryfonidou et al., 2003). The procedures were approved by the Utrecht University Ethics Committee for Animal Care and Use (DEC 99070-cs/b4). In this study, five Great Danes (median body weight 24.9±2.4 kg) and five Miniature Poodles (median body weight 3.5±0.7 kg) were raised until the age of 21 weeks on a balanced diet (Tryfonidou et al., 2003).
The bone marrow derived MSCs and articular cartilage cells (ACs) were obtained from dogs that were euthanized for unrelated experiments that were approved by the Utrecht University Ethics Committee for Animal Care and Use.

Next generation sequencing(NGS) (Performed by F.R. Riemers and M.A. Tryfonidou)

Growth plate isolation

For the NGS the growth plate (consisting of the reserve, proliferative, and hypertrophic zones) and adjacent metaphysis (1 mm) of the 9th rib were collected at 21 days after euthanizing the dogs. These samples were immediately frozen in liquid nitrogen, and stored at -70°C until further processing. Of the growth plates of 3 Great Danes and 3 Miniature Poodles 6-10 60µm cryo-sections were cut and stained with hematoxylin (Vector Laboratories, H3404) for 15 seconds in order to increase visibility of the different growth plate zones. Subsequently, from each section the reserve zone, proliferative zone, hypertrophic zone and bone were dissected under 25-50x magnification stereo scope, resulting in a total of 24 samples that were obtained.

RNA sequencing library preparation

RNA was isolated from each of the dissected growth plate zones using the RNeasy microkit (Qiagen) according to manufacturer's instructions, using 17µl of RNAse free water to elute the RNA.
The total RNA concentration was measured using a nanodrop ND-1000 spectophotometer (Isogen).The RNA integrity (RIN) was determined with the bioanalyzer 2100 (Agilent technologies) using a RNA nano chip and in addition, a small RNA profile was collected using the Small-RNA-chip. All RIN values of the samples used were higher than the value of 7, and the small RNA analysis showed that the small RNA fraction of the samples contained 20% miRNA.

The ribosomal RNA was depleted using the Ribominus eukaryote kit (Invitrogen) using 500-2000ng of total RNA as input. The rRNA depleted sample was used for whole transcriptome library preparation using the SOLiD total RNA-seq kit (Ambion/Life technologies) according to a modified low input protocol as outlined in the SOLiD® Total RNA-Seq Kit manual (4452437 Rev. B July 2011).

The library's size distribution was assessed using a high sensitivity DNA-chip on the bioanalyzer 2100 (Agilent) and the concentration was determined using the Qbit (Invitrogen). The libraries were clonally amplified in a emulsion PCR and after enrichment sequenced on a SOLiD 5500 sequencer.

Data analysis

Sequencing reads were mapped to the canine genome (CanFam 3.1_75) using the Burrows-Wheeler Aligner (BWA-0.5.9). Exonic mapped reads were counted using HTSeq–count and the total reads per gene were determined. The gene counts were analyzed using edgeR (v3.8.2, (McCarthy, Chen, &

Smyth, 2012; Robinson, McCarthy, & Smyth, 2009)) first the reads were filtered, and only genes with a CPM of >2 in at least 4 samples were kept, leaving 12851 of 24580 genes for the analysis.

Normalization factors were calculated to scale the raw library sizes (ranging from 1.5 million 8.5 million reads.) using TMM (Robinson & Oshlack, 2010). After estimating the dispersion a general linear model was fitted and the differential expression calculated for the contrasts of interest. Differentially expressed genes with a p-value < 0.05 using Benjamini-Hochberg FDR determination were considered significant.

Quantitative PCR validation of NOTCH expression in the growth plate zones

Mean total RNA of the different zones was extracted as described previously. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Biorad) according to the manufacturers protocol.
The dog specific primers were designed using PerlPrimer v1.1.14 on Ensembl annotated transcripts, and the amplicon was tested for secondary structures using MFold. The optimum temperature for each primer was determined using gradient polymerase chain reactions (PCRs). Subsequently, the primer specificity was validated in silico (BLAST specificity analysis) and empirically by observing the melt curves of the PCR products. The primers used for the experiments were *Neurogenic locus notch homolog 1,2* and *4* (*NOTCH1* and *NOTCH4)* and *JUN*, a gene that encodes the C-JUN protein, also known as *Activator protein 1 (AP-1)* (Supplemental file S-1). Quantitative PCR (qPCR) analysis was performed using a BioRad CFX-384 cycler and IQ SYBRGreen SuperMix (BioRad, Veenendaal, the Netherlands). *Hypoxanthine guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH),* and *ribosomal protein S19 (RPS19)* were used as reference genes for normalization of the data. Analysis of the qPCR data was performed with CFX Manager™ software (Bio-Rad). Relative expression of the genes of interests was estimated using the efficiency-corrected delta Ct (ΔCt) method using the average relative amount of the reference genes to normalize the data. All genes were compared to one common value, created by the mean values of all conditions.

Immunohistochemical staining of Notch4

Biopsy samples of the 9th rib of 4 Great Danes and 4 Miniature Poodles were cut longitudinally into slices, containing costal and growth plate cartilage, and part of the adjacent metaphysis. The slices were fixed in 4% neutral buffered formalin (NBF (Klinipath)) , decalcified in 10% EDTA and embedded in paraffin. 5 µm thick paraffin sections were cut, mounted on KP Plus slides (Klinipath, PR-P-001) and dried at 37°C for 48 hours.
For immunohistochemical (IHC) analysis, sections were deparaffinised and hydrated after which the endogenous enzymes were blocked (Dako S2003, dual endogenous enzyme block) for 5 min at room temperature. Next, non-specific antibodies were blocked with normal goat serum, 1:10 dilution in phosphate buffer solution (PBS)). Hereafter the primary antibody against Notch 4 (rabbit polyclonal antibody, Aviva systems biology, ARP32052_P050) was diluted in PBS-Tween 0.1% (Tween20 (Boom)) to a concentration of 1:1500 and added to the sections. Sections were incubated overnight at 4°C. Normal goat serum was used as a negative control. After the overnight incubation, sections were incubated with peroxidase labelled polymer (Dako K4003, anti-rabbit) for 30 min at room temperature. A two times repeated 5 min-wash in PBS-Tween 0.1% was performed between each of these steps. Subsequently, chromogens were developed for 5 min using 3,3'-diaminobenzidine (DAB) peroxidase substrate solution (DAKO, K3468). After rinsing with demi water, sections were counterstained with hematoxylin QS solution (Vector Laboratories, H3404), dehydrated and covered using Vectamount (Vector Laboratories, H5000).
Images were obtained using a Olympus BX41 microscope (Olympus Europa GmbH) with Cell^F software (Olympus Europa GmbH) at a 100x magnification. Photos were stitched and analysed with

Adobe Photoshop CC (2014, Adobe Systems Software Ireland Ltd.). First the borders of the growth plates were determined (Figure 1). The border of the hypertrophic and proliferative zone was established by the expression of collagen X (Tryfonidou et al., 2010). The reserve zone was defined as the three to four cell layers above the first flattened chondrocyte at the base of a cell column. The total number and amount of positive cells for each zone were counted manually, and the percentage of positive cells was calculated.
Statistical analysis of the cell count was performed using the Wilcoxon rank sum test using R statistical software 2.15.

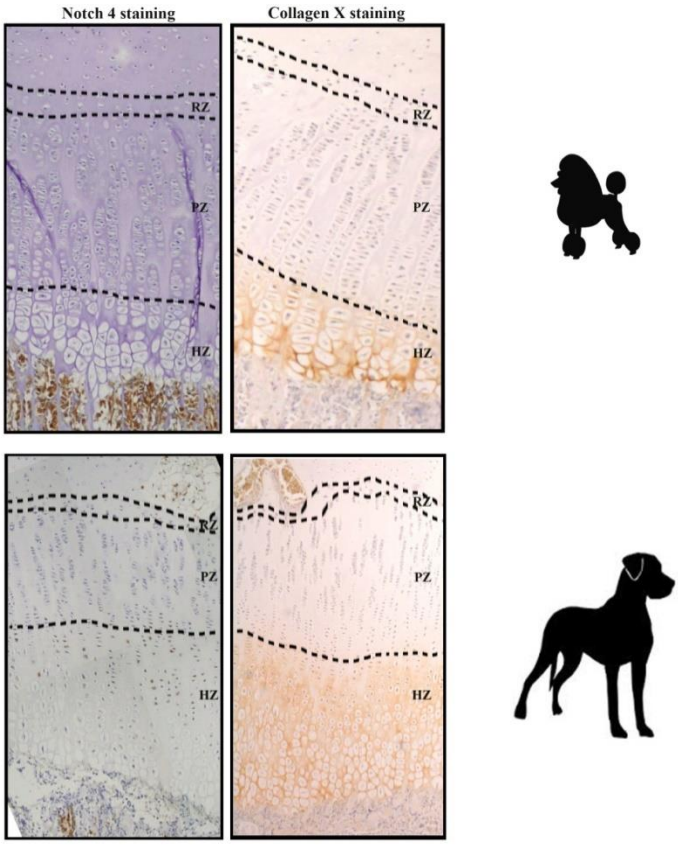


Figure 1: Determination of the growth plate zones in the NOTCH4 immunohistochemically stained sections. The border of the hypertrophic (HZ) and proliferative (PZ) zone was established by the expression of collagen X (dotted line). The proliferative zone was characterized by flattened columnar chondrocytes. And the reserve zone (RZ) was defined as three to four layers of cells above the first flattened chondrocyte at the base of a cell column (area between upper two dotted lines).

Cell culture

General cell culture

Canine bone marrow derived mesenchymal stromal cells (BM-MSC) and articular chondrocytes (AC) were cultured in expansion medium, consisting of a-MEM (Gibco; Life technologies) supplemented with 10% fetal bovine serum gold (FBS, high performance,16000-044; Gibco), 1% penicillin/streptomycin (PAA Laboratories) and 0.1 mM ascorbic acid (Sigma A8960). For the last experiment 1 ng/mL basic fibroblast growth factor (bFGF, AbD Serotec, PHP105) was added to the expansion medium in order to prime the cells for chondrogenic differentiation. BM-MSC were cultured at an initial density of roughly 4.000 cells/cm^2 in T175 culture flasks (Greiner Bio-One, CELLSTAR). Cells were cultured in humidified conditions at 37°C (5% CO_2) and expanded until 80% confluency in passage 2 was reached. Hereafter cells were used for the subsequent experiments.

Expression of NOTCH4 during normal chondrogenic differentiation of BM-MSC
Canine bone marrow derived mesenchymal stromal cells (BM-MSC) were isolated, expanded and differentiated towards the chondrogenic lineage as described previously (Malagola et al., 2016)(Malagola et al., 2016). Briefly, BM-MSCs of three donors were isolated and expanded in expansion medium until a confluency of >80% was reached in passage 2. Hereafter, cells were suspended in chondrogenic-inducing differentiation medium with or without the addition of 10 ng/ml TGF-β1. After 7 days, two pellets per condition per donor were collected for gene expression analysis by real time quantitative PCR (RT-qPCR).

Induction of NOTCH4 expression in *BM-MSC*
In order to evaluate the possibility to upregulate *NOTCH4* in BM-MSC to explore the possible use of these enhances BM-MSC in bone regenerative therapies, different hormones and/or growth factors or small molecules were added to the culture medium of BM-MSCs to stimulate upregulation of *NOTH4* expression.

Upregulation of NOTCH4 with glucocorticoid and growth factor stimulation
In the first experiment of this project, the addition of (the combination of) FGF-2 (bFGF, 1 and 10 ng/ml), dexamethasone (30 and 300 nM, Sigma D1756) and/or heparin (10 µg/ml, Leo Pharmaceutical Products BV, DG7794) to expansion medium was evaluated in duplo. BM-MSCs that reached passage 2 were plated at a density of 2000 cells/cm^2 in 24 well plates (Corning) and cultured in humidified conditions at 37°C (5% CO_2) until 80% confluence was reached and the expansion medium with additional factors was added. After 24 hours, cells were washed with Hank's Balanced Salt Solution (HBSS, Invitrogen) and 350 µl RLT (Qiagen) was added. Hereafter, cells were stored until qPCR analysis at -20°C.

Affecting NOTCH4 expression using the small molecules; Luteolin, Withaferin A and TPA
For the second experiment set-up the use of the small molecules Withaferin A, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and Luteolin to influence *NOTCH4* expression was investigated.
In a pilot experiment, 3 different concentrations of WA (2, 4 and 8 µM), TPA (5, 10 and 20 nM) and Luteolin (10, 50 and 100 µM) were added to the culture medium (consisting of expansion medium) of BM-MSCs originating from one donor. Cells were cultured at a density of 2000 cells/cm^2 in a 24 well plate at 37°C (5% CO_2). In addition, two control conditions were added; a control condition, consisting of BM-MSCs cultured in expansion medium, and a control DMSO condition, consisting of BM-MSCs cultured in expansion medium with 100 µmol/L DMSO. The DMSO control condition was added to distinguish the effects of the DMSO on *NOTCH4* expression, as 100 µmol/L DMSO is the concentration of DMSO in which the small molecules were dissolved before using. After 6 or 24 hours, cells were washed with HBSS, collected in RLT and stored at -20°C until further processing. All conditions were tested in duplo for both time points.

In the second experiment, the same concentrations of small molecules as described before were tested for their effect on the expression of *NOTCH4* in BM-MSCs and ACs of three different donors. In this experiment, cells were cultured in chondrogenic medium, consisting of DMEM (Invitrogen, 31966), 1% ITS+ premix (Corning, 354352), 0.4 mg/ml L-Proline (Sigma, P5607), 1% penicillin/streptomycin (PAA Laboratories), 0.1 mM ascorbic acid (Sigma A8960) and 1.25 mg/ml Bovine Serum Albumin (BSA, Sigma, A9418) at a density of 30.000 cells/cm^2 (BM-MSC) or 50.000 cells/cm^2 (AC) at 37°C (5% CO_2) in 24 well plates. All BM-MSC conditions were tested in duplo and the AC conditions in quadruplo. After 24 hours, the wells with BM-MSC and half of the wells with AC were washed with HBSS, collected in RLT and stored at -20°C until further analysis with qPCR. The other half of the

AC wells underwent six freeze-thaw cycles at -20°C after which 150 µl Tris-EDTA (TE) buffer (pH 7.4) was added in order to lyse the cells for DNA and glycosaminoglycan (GAG) content analysis. The DNA content was assessed using the Qubit™ dsDNA HS assay kit (Thermofisher scientific) according to the manufacturers protocols, using 20 µl of sample input. The GAG content was measured in order to evaluate the function of the AC, producing cartilage matrix proteins, under influence of small molecules. The GAG concentration of the samples was determined using a dimethyl methylene blue (DMMB) assay (Farndale, Buttle, & Barrett, 1986).

RNA isolation and quantitative qPCR
Total RNA of the cell culture samples was isolated using the RNeasy minikit (Qiagen 74134), according to the manufacturers protocol, including an on column DNAse step and using 32 µl RNAse free water to elute the RNA. RNA of the pellets was collected using the RNeasy microkit (Qiagen, 74004), using 17 µl RNAse free water to elute the RNA, after crushing the pellet with a pellet pestle (Argos technologies Inc, 9951-901) . The quantity of the RNA was determined using NanoDrop ND-1000 (Isogen Life Science, De Meern, the Netherlands).
After RNA isolation, cDNA was synthesized, with an input of approximately 500 ng RNA, and qPCR was performed as described earlier. *Heterogeneous nuclear ribonucleoprotein H (HNRPH), Signal recognition particle receptor (SRPR*) and *succinate dehydrogenase complex, subunit A (sDHA)* were used as reference genes for normalization of the cell culture data. No statistical analysis could be performed due to the small sample size and the absence of data for some of the conditions.

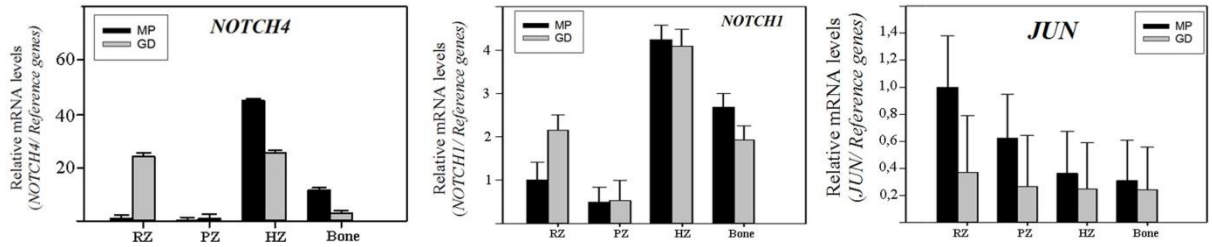
Results

Next generation sequencing(NGS)
As it is beyond the scope of this report, the results of the NGS analysis will be reported briefly with the main focus on Notch4 as this was the chosen target for this report.
Analysis of the NGS revealed a large amount of differentially expressed RNA elements of which, depending on the zone, 75-90 % was protein-coding. Interestingly, many other small or long non-coding RNA elements were also differentially expressed, suggesting that these elements may also be of interest in determining the pace of endochondral bone formation.
In the proliferative zone, a protein-coding RNA element, annotated as *NOTCH4* (ENSCAFG00000000791) was found to be upregulated in the GD compared with the MP with a fold change of 491. Furthermore, it was also found to be upregulated in the reserve zone of the growth plate, with a fold change of 3.

Although the relative expression is really high compared to other RNA elements, the absolute counts of the *NOTCH4* RNA element are quite low. The mean counts of *NOTCH4* transcripts in the reserve and proliferative zone of the GD were respectively 28 (range: 19-40) and 7 (range: 0-18) and for the MP this was 7 (range: 5-10) and 0 for the reserve and proliferative zone. The absolute transcript counts in the hypertrophic zone were higher for the both the GD (42 (range: 40-45)) and the MP (22 (range: 12-58)). Although there are two known transcripts of *NOTH4* (source: http://www.ensembl.org/, ID: ENSCAFG00000000791) , the transcript found in the GD and MP was the same.
For *NOTCH1* the mean counts of *NOTCH1* in the RZ, PZ and HZ of the GD were respectively 24 (range: 3-58), 36 (range: 32-42), and 22 (5-55). For the MP the *NOTCH1* counts were respectively 22 (range: 8-35), 27 (range: 7-49), and 15 (3-26).

Quantitative PCR validation of NOTCH expression in the growth plate zones
Expression of *NOTCH4* in the different growth plate zones in Great Danes and Miniature Poodles was validated with qPCR analysis. As shown in figure 2, gene expression of *NOTCH4* in the GD is mainly

found in the reserve and hypertrophic zone and is found to up regulated compared to the miniature poodle in the reserve and proliferative zone (Figure 2). In the MP *NOTCH4* expression is mainly found in the hypertrophic zone and in bone.
NOTCH1 mRNA expression levels are comparable in the GD and MP and are upregulated in the hypertrophic zone compared to the other zones (Figure 2). Expression of *JUN* seems to be generally higher in the MP compared to the GD although the gene expression is low in the growth plate.



Figuur 2: Relative mRNA levels of *NOTCH4, NOTCH1* and *JUN* in the different growth plate zones. Expression was measured in the reserve zone (RZ), proliferative zone (PZ), hypertrophic zone (HZ) and in the adjacent spongiosa bone (Bone) in the growth plate of the Great Dane (GD) and Miniature Poodle (MP). Gene expression was normalized by the average relative amount of the reference genes. All genes were compared to one common value, created by the mean values of all conditions.

Immunohistochemical staining of NOTCH4

IHC staining for NOTCH4 was found in four main regions; the endothelium of blood vessels in the reserve zone of the cartilage, within the chondrocytes of the growth plate, in the periosteal region and in the zone of ossification adjacent to the hypertrophic zone (Figure 3). In this last region, staining seemed to be mainly in the marrow zone of the bone and could be within the osteogenic cells or in the cells of the invading blood vessels.

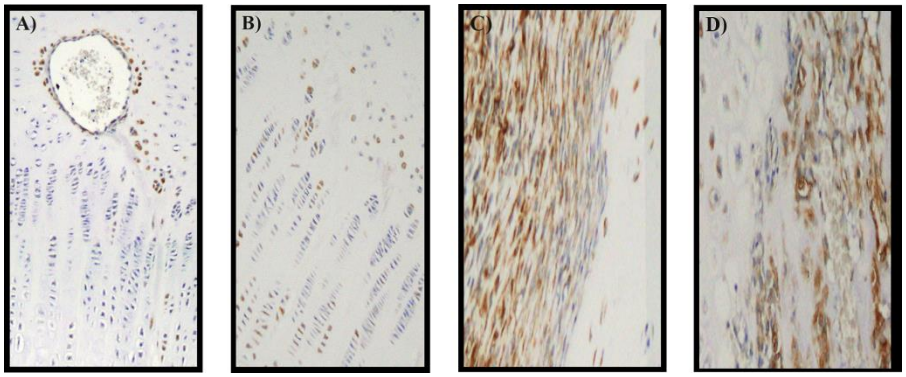


Figure 3: Positive staining for NOTCH4 in the growth plate of the Great Dane. Positive cells were found in the A) endothelium of blood vessels in the reserve zone and the surrounding chrondrocytes, B) the chondrocytes of the growth plate, C) the periosteum, and D) the zone of ossification

Within the growth plate, the highest percentage of NOTCH4 positive cells can be found in the reserve zone for both the GD as well as for the MP (Figure 4). In the reserve and proliferative zone as well as in the whole growth plate, the percentage of positive cells was significantly higher in the GD compared to the miniature poodle ($P < 0.05$) (Figure 4). Within the reserve zone, most of the positive chondrocytes were found in the neighborhood of blood vessels. In the GD, the positive chondrocytes surrounding blood vessels could also be found in the proliferative and hypertrophic zone.

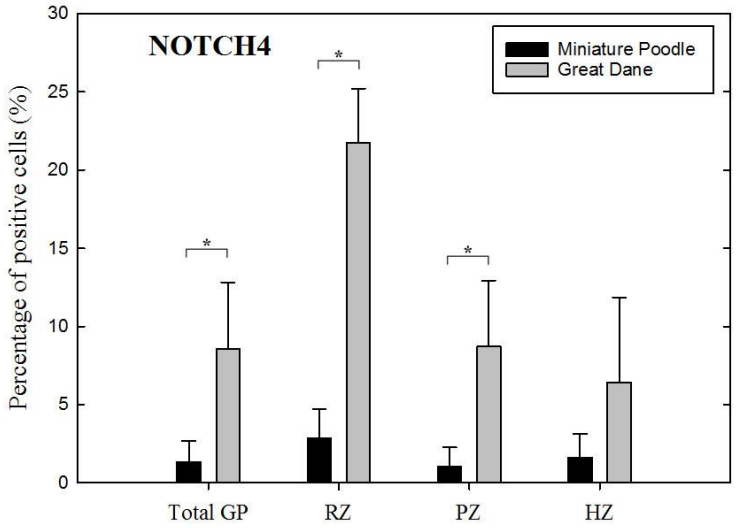


Figure 4: Percentage of cells with a positive staining for Notch4. The percentage of positive cells is given for each zone of the growth plate, the reserve zone (RZ), proliferative zone (PZ) and hypertrophic zone (HZ), and for the total growth plate (Total GP). The black bar provides the information for the Miniature Poodle (MP) and the grey bar for the Great Dane (GD). Significant differences ($P < 0.05$) between GD and MP are visualized with an asterix.

Cell culture

For the next part of this project, the expression of *NOTCH4* was investigated during the chondrogenic differentiation of BM-MSCs and after the induction of *NOTCH4* expression with growth factors or small molecules.

Expression of NOTCH4 during normal chondrogenic differentiation of BM-MSC

After 7 days of stimulating the BM-MSCs with chondrogenic differentiation medium, *NOTCH4* was not induced as no *NOTCH4* mRNA was detected by qPCR (data not shown) in both the control as well as the test condition to which TGF-β1 was added. Chondrogenic differentiation of the BM-MSCs was confirmed with qPCR and staining as is shown in *Malagola and Teunissen et.al. (*2016) (Malagola et al., 2016) (data not shown).

Upregulation of NOTCH4 with glucocorticoid and growth factor stimulation

In the first experiment, *NOTCH4* gene expression was attempted to be induced by the supplementation of the medium with heparin, FGF-2 and/or dexamethasone. None of the conditions showed an upregulation of *NOTCH4* expression compared to the control group (Figure 5). In the conditions with only heparin, FGF (1 ng/ml) or dexamethasone no expression of *NOTCH4* was detected. In addition no expression could be found in the condition with heparin and 10 ng/ml FGF and the condition with 1 ng/ml FGF and 30 nM dexamethasone. *NOTCH1* expression was upregulated compared to the control condition in all conditions with 10 ng/ml FGF and in the 1 ng/ml FGF conditions without dexamethasone (Figure 5).

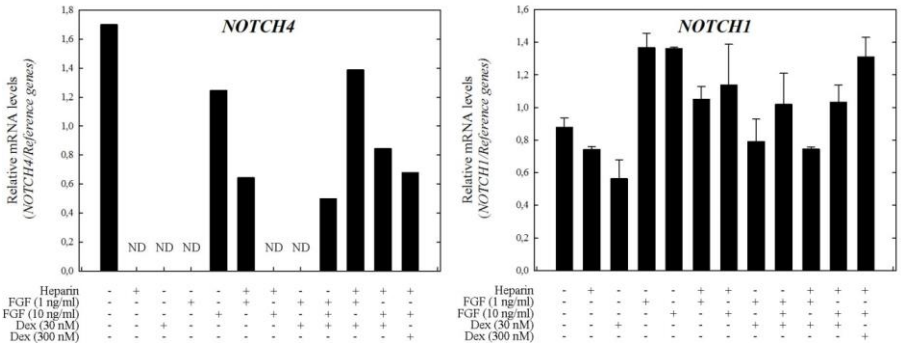


Figure 5: The relative gene expression of *NOTCH4* and *NOTCH1*. The relative gene expression of *NOTCH4* and *NOTCH1* , as provided by quantitative PCR analysis, in BM-MSCs after stimulation with different concentrations heparin (10 µg/ml), fibroblast growth factor 2 (FGF (1 or 10 ng/ml)) and/or dexamethasone (Dex (30 or 300 nM)). Gene expression was normalized by the average relative amount of the reference genes. All genes were compared to one common value, created by the mean values of all conditions. If no mRNA was detected a ND was inserted.

Affecting NOTCH4 expression using the small molecules; Luteolin, Withaferin A and TPA

After 6 hours, expression of *NOTCH4* was found in four conditions; 4 µM of WA, 10 nM and 20 nM of TPA and 100 µM of Luteolin (Figure 6). The expression of *NOTCH4* was higher with 10 nM of TPA compared to the 10 nM of TPA. At 24 hours, expression of *NOTCH4* was only found in the control condition.

NOTCH1 expression was found to be higher in the control and DMSO group at 6 hours compared to the 24 hours (Figure 6). The expression of *NOTCH1* at 6 hours after stimulation with WA was not upregulated compared to the control conditions. However, it increased with the concentration of WA. The highest expression of *NOTCH1* after supplementation with TPA at 6 hours was found in the lowest concentration of TPA (5 nM). At 24 hours, the expression of *NOTCH1* when induced with WA increased with the concentration of WA but was only higher compared to the control conditions for the highest concentration (of 8 µM). The expression after stimulation with TPA was roughly the same for every concentration of TPA and was not up regulated compared to the control conditions. Expression of *NOTCH1* under influence of Luteolin was only found with the lowest concentration but was not higher than the expression in the control conditions.

Expression of *JUN* was upregulated compared to the control conditions after the stimulation with WA after 6 hours with the highest concentration of WA (8 µM) and after 24 hours with a concentration of 4 µM WA (Figure 6).

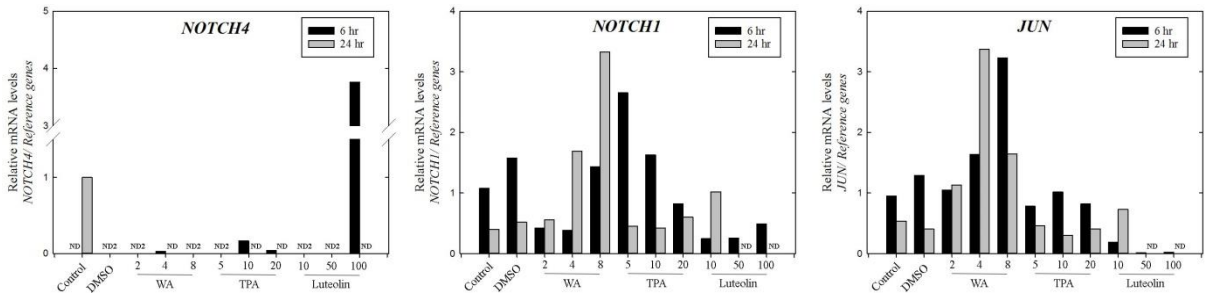


Figure 6: The relative gene expression of *NOTCH4, NOTCH1* and *JUN* after stimulation with the small molecules WA, TPA and Luteolin. The relative gene expression of *NOTCH4*, *NOTCH1* and *JUN*, as provided by quantitative PCR analysis, in BM-MSCs after stimulation with different concentrations WA (2, 4 and 8 µM), TPA (5, 10 and 20 nM) and Luteolin (10, 50 and 100 µM) for 6 hours (black bar) or for 24 hours (grey bar). Gene expression was normalized by the average relative amount of the reference genes. All genes were compared to one common value, created by the mean values of all conditions. If no mRNA was detected in of two cell types, ND was inserted, if both cell types did not show any expression ND2 was inserted.

After the pilot experiment with one donor in one cell type, the experiment was repeated for one time point but with two different cell types. For each cell type three donor animals were used. In addition to the BM-MSCs, ACs were used to investigate the effect of the small molecules on chondrocytes, the cell type that is also present in the growth plate.
Expression of *NOTCH4* after 24 hours was induced in only one BM-MSC donor with 4 µM and 8 µM WA (Figure 7). In the ACs, expression of *NOTCH4* was induced more conditions, but only upregulated after stimulation with 50 µM or 100 µM of Luteolin compared to the control (DMSO) condition (Figure 7).
In both cell types, Luteolin (10 µM and 50 µM) seems to induce *NOTCH1* expression (Figure 7). The expression is higher in the BM-MSC which also seems to be upregulated compared to the control conditions. In addition, WA (4 µM and 8 µM) seems to induce *NOTCH1* expression in both cell types. However, the dose response effect varies between cell type; for the BM-MSC the optimal concentration seems to be 4 µM while for the AC it is the 8 µM concentration of WA.
The same dose response effect is seen for both cell types regarding the stimulation of *JUN* by WA (Figure 7), where for both cell types the expression in addition seems to be upregulated compared to the control conditions. All TPA concentration seem to increase *JUN* expression compared to the control conditions for the AC while for the BM-MSC it seems to decrease expression.

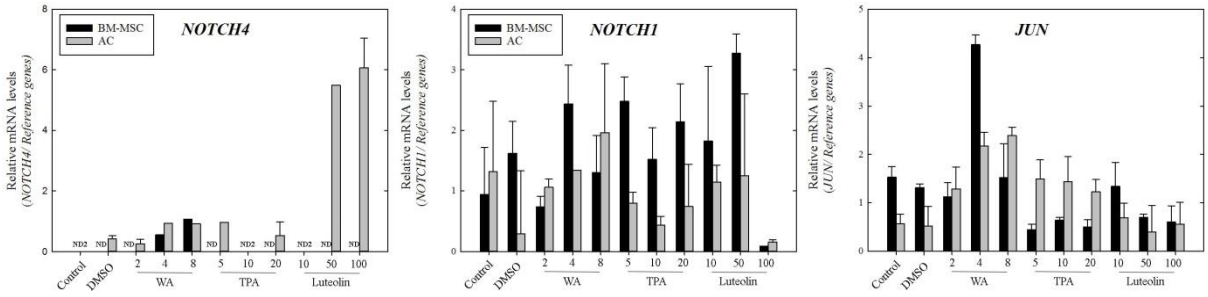


Figure 7: The relative gene expression of *NOTCH4, NOTCH1* and *JUN* after stimulation of BM-MSC and AC with the small molecules WA, TPA and Luteolin. The relative gene expression of *NOTCH4*, *NOTCH1* and *JUN*, as provided by quantitative PCR analysis, in BM-MSCs (black bar) and ACs (grey bar) after stimulation with different concentrations WA (2, 4 and 8 µM), TPA (5, 10 and 20 nM) and Luteolin (10, 50 and 100 µM) for 24 hours. Gene expression was normalized by the average relative amount of the reference genes. All genes were compared to one common value, created by the mean values of all conditions. If no mRNA was detected in of two cell types, ND was inserted, if both cell types did not show any expression ND2 was inserted. Standard deviations are provided by error bars for the the values consisting of more than one sample.

Analysis of the glycosaminoglycan (GAG) and DNA content showed a upregulation of GAG content per sample compared to the control conditions after stimulation of the cells with 50 µM Luteolin (Figure 8). DNA content was increased in the AC samples that were induced with TPA compared to the control conditions. In both the WA as well as the Luteolin stimulated samples, a dose dependent decrease in DNA content per sample was observed. Due to this decrease in DNA content, a small up regulation of the GAG/DNA content was seen in samples stimulated with the 4 µM and 8 µM WA concentrations.

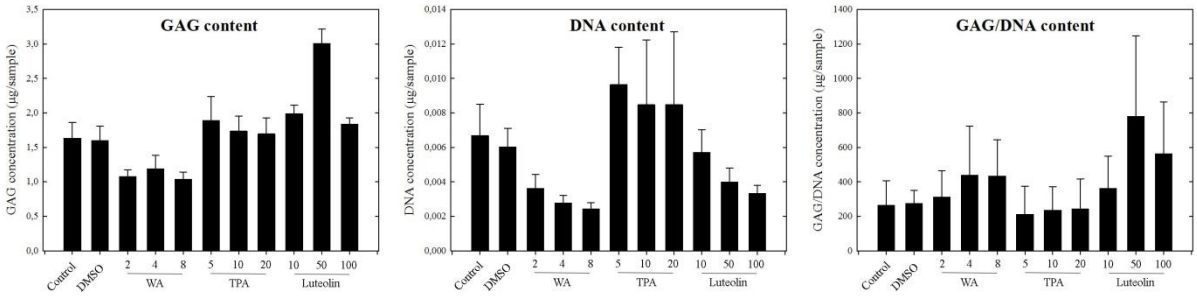


Figure 8: Glycasaminoglycan (GAG) content, DNA content and GAG/DNA content of ACs after stimulation with the small molecules WA, TPA and Luteolin. The GAG , DNA , and GAG/DNA content after stimulation with different concentrations WA (2, 4 and 8 µM), TPA (5, 10 and 20 nM) and Luteolin (10, 50 and 100 µM) for 24 hours is given in µg/sample. Standard deviations are provided by error bars for the the values consisting of more than one sample.

Discussion

The purpose of this project was to validate one of the results found with NGS of the different zones of the Great Dane and Miniature Poodle in order to gain a better understanding of the local growth factors that influence the pace of endochondral bone formation.
NOTCH4 seemed to be an interesting candidate as it was found to be up regulated in the GD compared with the MP with a fold change of 491 in the proliferative zone. However, this is a relative up regulation of expression. The absolute counts of this transcript were very low (7 (0-18) in the GD compared to 0 in the MP). This clarifies the high relative expression differences between the GD and the MP; with so few counts even 1 count extra in a GD growth plate zone accounts for a large increase in differential expression value. The low expression of NOTCH4 in the proliferative zone was confirmed by qPCR analysis of NOTCH4 in the different zones of the growth plate. It is therefore worth debating what the biological relevance is of a gene that is expressed in low concentrations. *SOX9* for example, a gene important for the development of the growth plate, has an average count in the proliferative zone of 224 transcripts and *COL2a1,* an important cartilage matrix component, an average count in the proliferative zone of almost 40000. Interestingly, *NOTCH1* is differentially expressed in the reserve zone with a fold change of 3,1 with an absolute count of 24 transcripts in the GD. Although this could be considered to be in the same range of moderate to low counts in which *NOTCH4* can be found, the biological relevance of Notch1 in the reserve zone is known. Murine studies showed that *Notch1* is expressed in early mesenchymal condensations where it promotes chondrogenic specification, although over-expression of the Notch 1 intracellular domain or Dll1 inhibits chondrogenic proliferation and differentiation by inhibiting *SOX9 (Mead & Yutzey, 2012).* Therefore, it would be interesting to deepen more into the role of *NOTCH4* in the growth plate.

The next step to investigate the role of *NOTCH4* in the growth plate was to study the localization of NOTCH4 protein in the different zones of the growth plate. In agreement with the NGS data, a significant higher amount of NOTCH4 positive cells was found in the reserve and proliferative zone of the GD compared with the miniature poodle. Although this is an interesting finding, the exact localization of the NOTCH4 positive chondrocytes in the reserve zone was remarkable: surrounding the blood vessels in the reserve zone, which in fact consisted of NOTCH4 positive endothelial cells. This pattern is repeated in the rest of the zones where the chondrocytes surrounding the blood vessels in the proliferative and hypertrophic zone also show positive staining for NOTCH4. An interesting theory could therefore be that there is an interaction between the cells of the endothelium and the chondrocytes of the growth plate influenced by the Notch signaling pathway. In line with these findings are the results of a study by *Dishowitz et. al.* (2012),where although *NOTCH4* was the least expressed Notch receptor during both endochondral as intramembranous bone formation, it showed

the greatest fold change among all receptors at the 10th day post fracture compared to day 0 (Dishowitz, Terkhorn, Bostic, & Hankenson, 2012). It could be that *NOTCH4* expression is important in the vascularization during endochondral bone formation, which is at this moment a major problem in the reconstruction of large bone defects.

Unfortunately the growth factors and small molecules used were not able to induce expression of *NOTCH4* after 1 day of stimulation in BM-MSCs as evaluated with qPCR. In the ACs, Luteolin was able to induce NOTCH4 expression. However, according to the study of *Reipas et. al.* Luteolin should inhibit *NOTCH4* (Reipas et al., 2013). The deviations of the results of this study compared to the other studies could be due to the culture time but is possible more related to the fact that these cell types are not induced by the same molecules or via the same pathways as the cell types used in the described experiments. For example, in the study of Wu et al. (2007) it is proposed that in endothelial cells the glucocorticoid receptor (GR) and activator protein 1 (AP-1) synergistically activate *Notch4* transcription (Wu & Bresnick, 2007). However, it is suggested that activation by these components is only possible in endothelial cells due to the cell-specific histone modification pattern of the *NOTCH4* promoter and cell-type-specific AP-1 complexes within the *NOTCH4* promoter (Wu et al., 2005). There are still some other candidates to test that may be able to enhance *Notch4* transcription in BM-MSC or chondrocytes. For example, it was found that tumor necrosis factor (TNF) induces *Notch4* mRNA in arthritic, but not normal, synovial fibroblasts (Ando, Kanazawa 2003). In addition, vascular endothelial growth factor 121 (VEGF121) has been found to modestly increase *Notch4* and *Notch1* mRNA in human umbilical vein endothelial cells (Liu, Shirakwa, Li 2002). However, the question remains whether the use of growth factors, hormones and/or small molecules is the most optimal method for the stimulation of *NOTCH4* expression. Both the growth factors and the small molecules were not specific for *NOTCH4* because they also induced the expression of *NOTCH1*. When investigating the effects of an overexpression of *NOTCH4*, it is undesirable if the products that are used also induce the expression of other genes, as it will not be possible to assign the results to the gene of interest. Therefore it would be better to overexpress the *NOTCH4* gene directly by transfecting cells with a *NOTCH4* plasmid. In addition, the effect of the small molecules on the cell viability should be observed as the amount of DNA decreased after stimulation of the cells with WA and Luteolin, which could mean that these molecules may increase the amount of cell death.

Conclusion

The purpose of this study was to investigate the role of *NOTCH4* in the growth plate, as this gene was found to be differentially expressed in the growth plate of GD compared with the MP with NGS with a fold change of 491 in the proliferative zone. The presence of different concentration of NOTCH4 protein in the growth plate of the GD compared to the MP validated this differential gene expression on the protein level. Together with the interesting localization of many NOTCH4 positive chondrocytes, surrounding the blood vessels, it would be definitely interesting to investigate the role of Notch4 in the growth plate further. Unfortunately *NOTCH4* expression could not be induced consistently by any of the growth factors or small molecules used in this study and therefore other methods have to be used to study the effect of *NOTCH4* signalling in BM-MSCs and articular chondrocytes.

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